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(54) Title: DERIVATIVES OF LINCOSAMINIDE ANTIBIOTICS

(57) Abstract

The invention relates to a new pharmaceutical form of a medicament of the formula: Lincosaminide-Radical-Carrier in which "Lincosaminide" represents an antibiotic lincosaminide; "Radical" represents a radical bound through a carboxylic function to the hydroxyl group of the antibiotic with another functional group susceptible to allow the attachment of the "Lincosaminide-Radical" complex to a carrier; and "Carrier" represents a peptide, a protein, or another macromolecule with free amino or carboxyl group(s) which allows the "Lincosaminide-Radical-Carrier" complex to specifically bind to cells. The "Lincosaminide-Radical-Carrier" complex is useful to treat bacterial infections and is particularly useful to treat bovine mastitis.

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DERIVATIVES OF LINCOSAMINIDE ANTIBIOTICS

Field of the Invention

This invention relates to new pharmaceutical derivatives of lincosaminide antibiotics. These novel derivatives provide for intercellular delivery of the antibiotic and are particularly useful to treat bovine mastitis.

Background of the Invention

Lincosaminides are antibiotics active against Gram (+) bacteria including clinical isolates of Staphylococcus aureus. Lincosaminides known in the art include lincomycin, clindamycin, pirlimycin and pharmacologically active derivatives of these antibiotics. In bovine mastitis and other diseases, the infecting bacteria accumulate in phagocytes such as polymorphonuclear neutrophil leukocytic cells or macrophages and are not affected by antibiotic treatment because the antibiotic only penetrates the phagocyte very slowly. In order to improve the rate of penetration of lincosaminides in phagocytes, the antibiotic is linked to suitable macromolecular carriers. These cells are particularly susceptible to antibiotic-carrier complexes which enter the phagocytes by endocytosis because phagocytes have high endocytotic activity. This invention allows the antibiotic-carrier complex to penetrate the phagocyte cell and then release the antibiotic in an active form within the cell in order to eradicate the bacteria inside these cells.

Information Disclosure

Derivatives of lincosaminides are known in the art. Sinkula, et al., Chemical Modification of Clindamycin: Synthesis and Evaluation of Selected Esters, J. Pharm. Sci., 1106-1111 (1973) disclose a series of 2- and 3- monoesters and some 2,3-dicarbonates of the lincosaminide, clindamycin. Arena, et al., On the Pharmacological Properties of Two Derivatives of 7-Cl-7-deoxylincomycin: Clindamycin-2-palmitate, Clindamycin-2-phosphate, Drugs Exptl. Clin. Res., 79-86 (1977) also discloses derivatives of the lincosaminide, clindamycin. U.S. Patent 3,326,891 discloses lincomycin acylates wherein the acyl derivative is a carboxylate radical containing not more than 12 carbons or a halo-, nitro-, hydroxy-, amino-, cyano-, thiocyano-, or lower alkoxy substituted hydrocarbon carboxylate of not more than 12 carbon atoms. U.S. Patent 3,580,904; 7-Halo-7-Deoxy-Lincomycin Derivatives, discloses lincosaminides substituted at

the 2 position with a carboxylate radical of not more than 18 carbon atoms or a halo-, nitro-, amino-, cyano-, thiocyano-, or lower alkoxy substituted hydrocarbon carboxylate radical of not more than 18 carbon atoms. U.S. Patent 4,376,765; Medicaments, Their Preparation and Compositions Containing Same, discloses new pharmaceutical forms of known drugs wherein the drug is attached to a proteinaceous macromolecule by a peptide chain spacer arm. Gilbertson and Stryd, Radioimmunoassay for Clindamycin, Clinical Chemistry, 828-831 (1976) discloses a clindamycin bound to a protein by a succinic spacer arm.

Summary of the Invention

This invention encompasses chemical complexes and a method of treating bovine mastitis by administering to a cow an effective amount of a chemical complex represented by the formula

LINCOSAMINIDE-RADICAL-CARRIER

wherein LINCOSAMINIDE is an antibiotic lincosaminide; wherein RADICAL is an organic compound attached to a hydroxyl group of the LINCOSAMINIDE by a carboxyl group and attached to the CARRIER by a carboxyl or amino group; and wherein CARRIER is an organic compound capable of transporting or directing said complex to a specific cell type.

Detailed Description of the Invention

Methods for making lincosaminides are known to those skilled in the art. The preparation of the following lincosaminides are described in the listed references; lincomycin, U.S. Patent 3,086,912, clindamycin, U.S. Patent 3,496,163, and pirlimycin, U.S. Patent 4,278,789.

The present invention relates to derivatives of lincosaminides in which an appropriate radical has been attached to a hydroxyl group of the antibiotic and to a carrier molecule. The radical can be any multifunctional molecular compatible with known pharmaceutical usage provided it meets the following requirements: a) It carries a carboxyl function through which it is attached to the lincosaminide by substitution of one of the hydroxyl functions on the drug; b) It carries a second carboxyl group or an amino group attached to the appropriate carrier having free amino or carboxyl groups using well established condensation reaction methods; and c) It releases the drug in an active form from the lincosaminide-radical-carrier complexes by hydrolysis of the lincosaminide-radical linkage under conditions compatible with the survival of eukaryotic cells. The

carrier can be a peptide or a protein which will deliver the drug to cells which possess recognition sites or affinity for such carriers and will internalize the drug in these cells using biological mechanisms that differ from simple diffusion.

The present invention includes examples of radicals and carriers which fulfill these three essential conditions. The rate of release of the lincosaminide can be varied according to the pH and to the nature of the radical used.

The attachment of the lincosaminide to the radical is performed by condensing a suitable anhydride or mixed anhydride with a suitable lincosaminide in the presence of a tertiary amine, such as triethylamine, in a suitable solvent, such as dichloromethane. The lincosaminide is preferentially protected on the amino group by reacting the amine with di-t-butyl carbonate and on the hydroxy groups at the 3 and 4 positions by reacting the glycoside with 2,2-dimethoxypropane in the presence of acid in dimethylformamide. After the radical is attached the protecting groups are removed. When the radical is a peptide, an appropriate N-protected peptide is attached to the protected lincosaminide in the presence of dicyclohexylcarbodiimide. After the radical is attached the protecting groups are removed. The methods described above are well known in the art and other known condensation methods could be used to attach the radical to the lincosaminide.

The attachment of the lincosaminide-radical to the carrier is performed in two different ways using carbodiimides as coupling agents. In the first way, the attachment is performed in a monophasic reaction mixture using diethylaminopropyl carbodiimide as coupling agent between the carrier and the lincosaminide-radical. In the second way, the attachment is performed in a biphasic reaction mixture using dicyclohexylcarbodiimide as coupling agent between the carrier and the lincosaminide-radical. The requirements for protecting groups in the above processes are well recognized by one skilled in the art of organic chemical synthesis and suitable protecting groups would be used in the processes as required following established procedures well known in the art. It is recognized that conditions for introduction and removal of protecting groups should not detrimentally alter any other groups in the molecule.

The preparation a representative protein carrier, rat monoclonal

immunoglobulin G, is described below.

DMEM 10% serum is used for the culture of the IR983F myeloma. This medium is prepared by using Dulbecco's Modified Eagle's Medium (Gibco Ltd., Paisley, Scotland, UK; medium no. 074-1600) supplemented with (for one liter) 3.5 g glucose, 100 mg streptomycin, 60 mg penicillin, 50 ml fetal calf serum, 50 ml horse serum and 10 ml of Non-Essential Aminoacids Solution (Gibco Ltd., Paisley, Scotland, UK; solution no. 043-1140).

DMEM Hepes is used for the fusion experiments. This medium is a serum-free DMEM medium supplemented with 10 mM Hepes pH 7.2-7.4.

PEG solution is made of polyethyleneglycol 4000 (Merck AG, Darmstadt, W. Germany) 62.64% (w/v) in DMEM Hepes medium supplemented with 12.9 ml dimethylsulfoxide.

DMEM HAT-serum rich medium is used for the selective growth of the hybridoma cells. This medium is made of DMEM 10% serum medium enriched with 5% fetal calf serum and supplemented with 13.6 mg/l hypoxanthine, 3.88 mg/l thymidine and 0.178 mg/l aminopterine.

For all cultures performed in 10% CO_2 atmosphere, 1.5 g $NaHCO_3$ is added per liter of the culture media described above.

PBS pH 7.4 (phosphate buffered saline) is routinely used for washing the cells. This solution is made of 137 mM NaCl, 2.7 nM KCl, 8 mM Na_2PO_4, and 1.5 nM KH_2PO_4.

Buffered EDTA is 1.5% EDTA in 13.2 mM phosphate - 0.7% NaCl adjusted to pH 6.8.

Bovine polymorphonuclear neutrophilic leukocytes (PMN) are isolated from cow blood following the procedure of Carlson and Kaneko, Isolation of Leukocytes from Bovine Peripheral Blood, Proc. Soc. Exp. Biol. Med., 853-856 (1973). Blood (10 volumes) is collected on a solution of buffered EDTA (one volume). After centrifugation at 1000 g for 15 minutes, the plasma and the buffy coat are discarded. The pellet (containing the erythrocytes and the bulk of the PMN) is resuspended in 5 volumes of distilled water. Isotonicity is restored after 30 seconds by the addition of one volume of 13.2 mM phosphate buffer pH 6.8-2.7% NaCl per 2 volumes of mixture. Leukocytes are collected by centrifugation at 200 g for 10 minutes. This fraction contains the PMN but is often contaminated with polymorphonuclear eosinophilic leukocytes. These are separated from PMN on a preformed Percoll® gradient described by Mottola, et al., Isolation

and Partial Characterization of the Plasma Membrane of Purified Bovine Neutrophils, Eur. J. Biochem., 341-346 (1980). A 68% suspension of Percoll® (Pharmacia AB, Uppsala, Sweden) in 0.9% NaCl adjusted to pH 7.3 is first centrifuged for 20 minutes at 21,000 rpm. The leukocyte suspension (in PBS) is then deposited on the top of this gradient, which is further centrifuged for 15 minutes at 10,000 rpm. The lowest band corresponding to the PMN (density approximately 1.094) is collected, whereas the eosinophils (equilibrating at density approximately 1.078) are discarded. The PMN's are washed with PBS pH 6.8 to remove the Percoll® and resuspended in DMEM medium.

The IR983F cell line derived from the Lou/C rat strain is used. This myeloma is a non-secreting, 8-azaquanine-resistant cell line adapted to in vitro growth in DMEM 10% serum according to procedures described by DeClercq, et al., Generation of Rat-Rat Hybridomas with the Use of the LOU IR 983 F Non-secreting Fusion Cell Line, Meth. Enzymol., 234-238 (1986).

These cells (mostly consisting of macrophages and fibroblasts) are obtained by peritoneal lavage of outbred Wistar rats, collected and cultivated in 96 well plates (Nunc A/S, Roskilde, Denmark, article no. 167008; 2 X 10^4 cells/well) in DMEM HAT serum rich medium.

Female, 2-month-old Lou/C rats are immunized with freshly isolated bovine PMN. Approximately 10^7 cells are injected in the footpad of the rat at days 0, 4, 8, 11. At day 14 the popliteal lymphnode is removed and the lymphnode cells mechanically dispersed.

The myeloma IR983F cells and the dispersed lymphnode cells are centrifuged separately for 5 minutes at 250 g and resuspended in DMEM-Hepes medium. IR983F cells and lymphnode cells are then counted and mixed at a ratio of one myeloma cell for 5 lymphnode cells. After centrifugation of the cell mixture, all medium is carefully discarded and one ml PEG solution is added dropwise to 2 X 10^7 cells over 90 seconds with gentle shaking. After 60 seconds of further gentle shaking, 2 ml of DMEM-Hepes is added dropwise over 90 seconds, followed by further progressive addition of 20 ml of the same medium. The suspension is centrifuged for 5 minutes at 180 g and the cells are carefully resuspended in dMEM HAT-serum rich medium (10^6 cell/ml). The cells are distributed in 96 well plates (0.1 ml per well) containing feeder layer cells.

Hybridomas (resulting from the fusion of lymphocytes with IR983F cells) are cultured in a 10% CO_2 atmosphere one to 3 weeks with change of half the culture medium (0.1 ml) every 3 to 4 days.

The culture medium is added to 5.10^6 bovine PMN. After one hour of incubation at 4°C, the PMN are washed with PBS and reincubated for one hour at 4°C with fluorescein-labeled or ^{3}H-methyl-labeled rabbit anti-rat immunoglobulins. After 3 washings with PBS the PMN are examined by fluorescence microscopy (for cells treated with fluorescent-labeled antibodies), or centrifuged, and their radioactivity measured by scintillation counting (for cells treated with ^{3}H-labeled antibodies). Hybridomas secreting anti-PMN antibodies are transferred to 24 well plates and further cultured in standard vessels.

Characterization (with respect to the classes and subclasses of rat immunoglobulins) is performed by 2-dimension immunodiffusion using specific antisera directed against rat IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, IgA, IgM, IgE, and IgD. Only clones secreting IgG types of immunoglobulins are kept for production. The clones selected in this step are propagated by culture in stationary vessels or by injection in the peritoneal cavity of Lou/C rats (with weekly transfer from animal to animal). The monoclonal immunoglobulins secreted in the ascitic fluid or in the culture supernatant are purified by passing the fluids on an immunosorbant column made of insolubilized monoclonal mouse antibodies directed against rat kappa light chain (MARK-1), Bazin, et al., Purification of Rat Monoclonal Antibodies, 638-652 (1986). MARK-1 antibodies are obtained from the ascitic fluid of Balb/C mice injected with a mouse hybridoma producing these antibodies and purified on an immunosorbant made of polyclonal immobilized rat immunoglobulins. After binding of the anti-PMN monoclonal immunoglobulin G to the immobilized MARK-1 antibodies, elution is performed with a 0.1 M glycine - 0.15 M NaCl buffer pH 2.8. The eluate is neutralized by addition of one M Tris buffer pH 8.0, and the purity of the preparation checked by standard electrophoresis.

The utility of the lincosaminide derivatives to act as antibiotic is demonstrated by the hydrolysis of the conjugate. In contrast to the corresponding known esters of galactose, the ester derivatives of pirlimycin release the free drug upon incubation at 37°C in aqueous media. After 24 hours at pH 7.0, the hydrolysis of 3 derivatives, pirlimycin-2'O-glycinate, pirlimycin-2'O-succinylglycin-

ate and pirlimycin-2'0-hemisuccinate, is about 60%, 45% and 30%, respectively. At 4°C, no or only negligible hydrolysis occurs, and the derivatives can therefore be safely handled and purified at that temperature. No detectable release of pirlimycin occurs in non-aqueous media. In aqueous media, addition of enzyme preparations susceptible to contain esterases (extract of lysosomes, macrophages) does not alter the rate or extent of hydrolysis, suggesting that this process is exclusively chemical.

About 2% to 23% of pirlimycin can be liberated from pirlimycin-2'0-hemisuccinate coupled to mannosylated serum albumin upon exposure of the conjugate to aqueous media at 37°C and at different pH. When the temperature is increased to 80°C, no further liberation is observed at either pH 5, 7 or 8 compared to the values observed after 96 hours at 37°C and pH 8.0. Thus, the conjugate probably contains approximately 25% of available pirlimycin and 75% of unavailable pirlimycin. Because pirlimycin-2'0-hemisuccinate is hydrolyzable to approximately 75%, it is unlikely that the pirlimycin which cannot be released from mannosylated serum albumin is not bound to the protein by its succinyl and/or its 2'OH group.

Various batches of pirlimycin-2'0-hemisuccinate rat monoclonal immunoglobulin G conjugate are incubated at 37°C and at pH 8. Between one and 6 pirlimycin molecules per molecule of conjugate are released after 96 hours. The nature of compound released is assessed by TLC (after revelation with orcinol/H_2SO_4 spray versus a TLC standard scale) and mass spectrum (detection of a molecular ion at 410).

The ability of the conjugate to enter cells is demonstrated by uptake in macrophages. Pirlimycin, N-^{3}H-glycyl-pirlimycin, and N-^{3}H-glycyl-pirlimycin-2'0-hemisuccinyl-mannosylated serum albumin are incubated in the presence of macrophages isolated from the mammary gland of a dry cow and cultured according to Bottcher, et al., Evidence for Receptor-mediated Endocytosis of Glycoconjucates by bovine mammary gland macrophages, Arch. Int. Physiol. Biochim., B54 (1987); or rabbit alveolar macrophages, isolated and cultivated according to Hoppe, et al., (1982). A shown in Table 1, conjugation of N-^{3}H-glycyl-pirlimycin to mannosylated albumin enhances its uptake by both types of macrophages.

Bovine mammary macrophages are incubated one hour in the presence of pirlimycin (labeled with ^{14}C), or N-^{3}H-glycyl-pirlimycin-

2'O-hemisuccinyl-mannosylated bovine serum albumin. Cells are collected, homogenized and the homogenate fractionated by isopycnic centrifugation in a sucrose gradient following the techniques used by Canonico, et al., J. Reticuloendoth. Soc., 115-138 (1979) and Renard, et al., Antimicrob. Agents Chemother., 410-416 (1987). After one hour of incubation, pirlimycin is almost entirely soluble, whereas the N-^{3}H-glycyl-pirlimycin-2'O-hemisuccinyl-mannosylated bovine serum albumin is largely recovered in fractions enriched in the lysosomal enzyme N-acetyl-beta-hexosaminidase. Thus, the conjugation of N-^{3}H-glycyl-pirlimycin-2'O-hemisuccinyl-mannosylated bovine serum albumin is allowed to drive it to the lysosomes of bovine mammary gland macrophages. In these experiments N-^{3}H-glycyl-pirlimycin is used for coupling to serum albumin rather than pirlimycin to allow for detection of the drug. Since it is unlikely that addition of a glycyl moiety to the coupled pirlimycin should markedly modify the behavior of the whole conjugate, the behavior of N-^{3}H-glycyl-pirlimycin-2'O-hemisuccinyl-mannosylated bovine serum albumin describes the potential fate of pirlimycin-2'O-hemisuccinyl-mannosylated bovine serum albumin.

The chemical complexes claimed in this invention can be used and administered in practicing the method claimed in this invention. Derivatives of the chemical complexes claimed in this invention which would be readily apparent to a manufacturing pharmaceutical chemist to be equivalent to the parent complex in properties such as formulation, stability, patient acceptance and bioavailability are included within the scope of this invention.

Those skilled in the art would know how to formulate the chemical complexes used to practice the method claimed in this invention into appropriate pharmaceutical dosage forms. The exact route of administration, dose, or frequency of administration would be readily determined by those skill in the art and is dependant on the age, weight, general physical condition, or other clinical symptoms specific to the patient to be treated.

Without further elaboration, those skilled in the art can practice the present invention to its fullest extent. The following detailed examples further describe how to make the chemical complexes claimed in this invention and how to treat bovine mastitis. These examples are merely illustrative and are not limitations of the

preceding disclosure. Those skilled in the art will promptly recognize appropriate variations from the examples. In each example, any compound claimed in this invention could replace the compound used in the particular example.

Example 1 Pirlimycin-3',4'-isopropylidene

A mixture of pirlimycin (2 g, 4,47 mmole), 2,2-dimethoxypropane (8,8 ml; 99 mmole) and toluene-p-sulphinic acid (460 mg) in dimethylformamide (25 ml) is stirred for 2 hours at room temperature. The mixture is then evaporated and the resulting syrup diluted with dichloromethane (50 ml) washed with 5% aqueous $NaHCO_3$ (20 ml) and water (3 x 20 ml) dried (Na_2SO_4) and concentrated to give the syrupy 3',4'-O-isopropylidene derivative. This material is then submitted to chromatography on silica column (Lobar Lichroprep Si 60, MERCK A.G., Darmastadt, W. Germany) in the solvent system Butanol-acetic acid-water (4:1:1). After evaporation and lyophilization of the eluate, 1.57 g (78 %) of pirlimycin 3',4'-O-isopropylidene is obtained. Rf (TLC) = 0.34 (methylene chloride-methanol-acetic acid 85:10:15). ^1H NMR (60 MHz, $CDCl_3$) spectra shows the appearance of two singlets corresponding to an area of 6 protons at 1.11 and 1.18 ppm (isopropylidene bridge). Mass spectra = 450.

Example 2 N-t-Butyloxycarbonyl-pirlimycin 3',4'-O-isopropylidene

To an ice solution of 2.5 g (5.4 mmole) of pirlimycin 3',4'-O-isopropylidene dissolved in 30 ml of a mixture of dioxane-water (2:1) and 10 ml of 1N NaOH (the pH of the solution is then 10.2), 12.29 g (5.94 mmole) of di-t-butylcarbonate ($(Boc)_2O$) is added dropwise during one hour. The pH is maintained at 10.2 by addition of 1N NaOH. At the end of the addition, the reaction mixture is vigorously stirred during one hour and more 1N NaOH added if necessary. When the reaction is complete (as evidenced by TLC eluted chloroform:methanol:acetic acid (85:10:5)), dioxane is evaporated, the resulting aqueous phase is extracted with ether (to eliminate unreacted ($(Boc)_2O$), and acidified to pH 2 by addition of 10% K_2SO_4. The N-Boc-pirlimycin-3',4'-O-isopropylidene is extracted with ethyl acetate and the organic layer washed with water (saturated with NaCl) and dried over $MgSO_4$. After evaporation of the solvent, the residual oil is submitted to a chromatography on silica column (LOBAR Lichroprep Si 60 Merck) in the solvent system petroleum ether-ethyl acetate (5:5). The residual oil is homogeneous in 3 different solvent

systems and is not further crystallized. Rf (TLC) = 0.48 (Petroleum ether:ethyl acetate, 1:1. ^1H NMR (60 MHz, $CDCl_3$) spectrum shows the appearance of a singlet of 9 protons at 1,55 ppm corresponding to the t-butyl group. IR spectrum (KBr) shows a new C=O band at 1650 cm^{-1} (carboxyl function of the t-Boc urethane group). Mass spectra = 550.

Example 3 N-t-butyloxycarbonyl-3',4'-O-isopropylidene-pirlimycin-2'O-hemisuccinate

N-t-butyloxycarbonyl-3',4'-O-isopropylidene pirlimycin (300 mg, 0.502 mmole) is dissolved in methylene chloride (10 ml) and 0.14 ml triethylamine (1.004 mmole) is added. A solution of succinic anhydride (502 mg, 5.01 mmole) in methylene chloride is subsequently added under anhydrous conditions. The reaction mixture is allowed to stand overnight at room temperature and thereafter refluxed for one hour. The solvent is evaporated under reduced pressure and the residue dissolved in methylene chloride. The solution is washed with 1N HCl and water and then dried over $MgSO_4$. After evaporation of the solvent, the oil obtained is analyzed by TLC (Rf = 0.17; petroleum ether:ethyl acetate, 3:7) and is found homogeneous. It is not further purified. IR spectrum (KBr) exhibits a new C=O band at 1645 cm^{-1}. Mass spectra = 650.

Example 4 Pirlimycin-2'O-hemisuccinate

N-t-butyloxycarbonyl-3',4'-O-isopropylidene-pirlimycin-2'O-hemisuccinate (200 mg) is dissolved in 25% trifluoroacetic acid in methylene chloride and stirred for 30 minutes at room temperature. The completeness of the reaction is checked by TLC of the reaction mixture in butanol:acetic acid:water (8:1:1). Evaporation under reduced pressure yields a syrup which is submitted to a chromatography on silica gel column (Lobar Lichroprep Si 60 MERCK) eluted with butanol:acetic acid:water (8:1:1). After lyophilization of the eluate, a white powder is obtained. Rf (TLC) = 0.42 (butanol:acetic acid:water, 4:1:1). ^1H NMR (60 MHz, $CDCl_3$) spectrum shows the disappearance of the singlets at 1.11 and 1.18 ppm corresponding to the 2 methyl of the isopropylidene bridge and of the 3 methyl of the Boc group. Mass spectra = 510.

According to the procedures described in Example 4, the following compounds can be attached to pirlimycin starting with the appropriate anhydride: glutaryl, 2,2-dimethylglutaryl, 3,3-dimethylglutaryl, 3-ethyl-3-methylglutaryl, 2-phenylglutaryl, 3,3-tetramethy-

leneglutaryl, phtalyl, 3-nitrophtalyl, isatoyl, N-methylisatoyl, 1,2-cyclohexanoyl, dimethylmaleyl, maleyl and citraconyl.

According to the procedures described in Example 4, the following compounds can be attached to pirlimycin through condensation of the appropriately N-protected amino acid or peptide and N-t-butyloxycarbonyl-pirlimycin-3',4'-O-isopropylidene in the presence of DCC followed by removal of the protecting groups with 25% trifluoroacetic acid in dichloromethane: glycyl, alanyl, leucyl, phenylalanyl, glycylalalanyl, undecanoyl, succinylglycyl, and glycylvalylleucylalanyl-phenylalanylglycyl.

Example 5 Pirlimycin-2'O-hemisuccinate-bovine serum albumin conjugate

Bovine serum albumin (200 mg, fraction V BSA, Merck AG, Darmstadt, W. Germany) is dissolved in 3 mM phosphate buffer pH 7.4 (2.5 ml). Diethylaminopropylcarbodiimide (EDC; 14 mg, Merck, AG, Darmstadt, W. Germany) is added and the reaction mixture is stirred for 30 minutes at 25°C. The amount of pirlimycin conjugated to serum albumin during the reaction is monitored by thin layer chromatography (eluted with butanol:acetic acid:water, 4:1:1, v/v; visualization with the orcinol/sulfonic acid reagent; Rf = 0.48 and 0 for pirlimycin-2'O-hemisuccinate and for the conjugate, respectively). The reaction mixture is dialyzed at 4°C for 8 hours against 3 mM phosphate buffer pH 7.4, and then 3 times 8 hours against distilled water. The undialyzed fraction is lyophilized and stored at -20°C.

According to the procedures described in Example 5, pirlimycin 2'O-hemisuccinate rat monoclonal immunoglobulin G conjugate is prepared.

Example 6 Mannosylated pirlimycin-2'O-hemisuccinate bovine serum albumin conjugate

N-t-butyloxycarbonyl-3'-4'-O-isopropylidene-pirlimycin-2'O-hemisuccinate (100 mg) is solubilized in 600 ul dimethylformamide (DMF) and the mixture is cooled at 0°C. Dicyclohexylcarbodiimide (36.26 mg; DCC, Merck AG, Darmstadt, W. Germany) is added to the mixture under stirring while the temperature is strictly maintained at 0°C. After exactly 3 minutes, N-hydroxysuccinimide (21.28 mg, Merck AG, in 0.2 ml DMF) is added dropwise to the reaction mixture and the reaction is allowed to proceed at 4°C for 24 hours, this time being necessary for forming a derivative capable of reacting with the

free amino groups of the carrier. At the end of this activation period, aliquots (0.05 ml) of this mixture are added every 5 minutes to a solution of bovine serum albumin (BSA fraction V, Sigma Chem., 327 mg in 35 ml of 0.1 N bicarbonate buffer pH 7.5). The reaction is performed for 3 hours at 25°C during which the biphasic DMF/water system is gently stirred. The reaction mixture is lyophilized and the resulting powder solubilized in 10 ml distilled water, and dialyzed 3 times 8 hours against distilled water. The residue is collected and stored at -20°C.

The conjugate is suspended in 10 ml trifluoroacetic acid:CH_2Cl_2 (1:3, v/v), and the mixture stirred for 30 minutes at 25°C. The reaction mixture is evaporated under vacuum and resuspended in 10 ml CH_2Cl_2. This procedure is repeated 2 times and the final residue suspended in 10 ml of toluene. After evaporation of the toluene under reduced pressure, the residue is dissolved in distilled water, filtrated and lyophilized. The resulting powder is stored at -20°C.

Mannosylation of the conjugate is performed by a method adapted from Kataoka, et al., Synthetic Neoglycoproteins: A Class of Reagents for Detection of Sugar Recognizing Substances, J. Histochem. Cytochem., 1091-1098 (1984). p-Aminophenyl-mannose (0.37 mmol; Sigma Chemical Co,., St. Louis, Mo.) is dissolved in 10 ml of 0.1 M hydrochloric acid and 0.01 M KBr in ice-cold water. Sodium nitrite (0.6 mmol, Merck AG, Darmstadt, W. Germany) in distilled water is added dropwise under stirring to form the diazonium salt of the derivatized sugar. The solution is stirred for 30 minutes and is thereafter added dropwise to 10 mg of an ice-cold solution of pirlimycin-2'O-hemisuccinate-bovine serum albumin conjugate (0.37 mmol in 0.01 M borate buffer pH 9.0) under constant stirring. The reaction is allowed to proceed at 0°C, for 3 hours, the pH being maintained at 9.0 by suitable addition of 0.5 M NaOH. To remove the unreacted mannose derivative, the neoglycoprotein is dialyzed at 4°C against 0.15 M sodium chloride for 8 hours, and then 3 times 8 hours against distilled water.

The product is purified by chromatography on Sephadex® G25 column eluted with 0.1 M ammonium acetate buffer pH 7. The fractions containing the conjugate are identified by monitoring the O.D. at 280 nm, collected, lyophilized and stored at -20°C. The total amount of mannose and pirlimycin conjugated to the albumin is assessed by

colorimetric assay, using the orcinol/sulfonic acid method (Francois, et al., 1962). Total protein concentration is measured by the method of Lowry, et al. (1951).

Pirlimycin-2'O-hemisuccinate mannosylated bovine serum albumin conjugate, pirlimycin-2'O-hemisuccinate-monoclonal-immunoglobulin conjugate, ^{3}H-glycyl pirlimycin-2'O-hemisuccinate mannosylated bovine serum albumin, and ^{3}H-glycyl-pirlimycin-2'O-hemisuccinate monoclonal immunoglobulin G conjugate are obtained with typical pirlimycin (or ^{3}H-glycyl-pirlimycin) to protein ratios indicated below.

Type of conjugate	Type of synthesis	Pirlimycin (or ^{3}H-glycyl-pirlimycin)-protein (molar ratio)
Pirlimycin-mannosylated serum albumin	monophasic	10.2, 14.8, 16.4*
	biphasic	6.4, 8.3, 10.1*
^{3}H-glycyl-pirlimycin-mannosylated albumin	monophasic	6.1
	biphasic	-
Pirlimycin-immunoglobulin G	monophasic	16, 17, 34*
^{3}H-glycyl-pirlimycin-immunoglobulin G	monophasic	12.6

* typical results of 3 experiments

Example 7 Mastitis ointment

One thousand gm of an ointment for the treatment of mastitis in dairy cattle is prepared from the following types and amounts of ingredients:

	Gm
Pirlimycin-mannosylated serum albumin	25
Methylprednisolone acetate	0.5
Light liquid petrolatum	300
Chlorobutanol anhydrous	5
Polysorbate 80	5

2% Aluminum monostearate in gelled peanut oil	400
White petrolatum q.s.	1000

Pirlimycin-mannosylated serum albumin and methylprednisolone acetate are milled with the light liquid petrolatum until finely divided and uniformly dispersed. The chlorobutanol, polysorbate 80, peanut oil gel and white petrolatum are heated to 120°F to form a melt and the liquid petrolatum dispersion stirred in. With continued stirring, the dispersion is allowed to cool (and congeal) to room temperature and is filled into disposable mastitis syringes in 10 gm doses.

Example 8 Mastitis Ointment

A 10 ml dose of an ointment for the treatment of mastitis in dairy cattle is prepared from the following types and amounts of ingredients:

Pirlimycin-mannosylated serum albumin	1.5 g
2% glycerol monostearate in gelled peanut oil	10 ml
Chlorobutanol	50 mg

The chlorobutanol and peanut oil are heated to form a melt (about 120°F) and Pirlimycin-mannosylated serum albumin is stirred in. The dispersion is allowed to cool to room temperature and is filled into a mastitis syringe.

Example 9 Mastitis Ointment

A 10 ml dose of an ointment for the treatment of mastitis in dairy cattle is prepared from the following types and amounts of ingredients:

Pirlimycin-mannosylated serum albumin	1.5 g
2% aluminum monostearate in gelled peanut oil	10 ml
Chlorobutanol	50 mg

The chlorobutanol and peanut oil are heated to form a melt (about 120°F) and Pirlimycin-mannosylated serum albumin is stirred in. The dispersion is allowed to cool to room temperature and is filled into a mastitis syringe.

Example 10 Mastitis Ointment

A 10 ml dose of an ointment for the treatment of mastitis in dairy cattle is prepared from the following types and amounts of ingredients:

Pirlimycin-mannosylated serum albumin	1.5 g
Benzyl alcohol	95 mg
Citric acid USP anhydrous	105 mg

Sodium carboxymethylcellulose USP medium viscosity	200 mg
Sodium hydroxide 10% solution	pH adjustment
Purified water	10 ml

The benzyl alcohol, citric acid, sodium carboxymethylcellulose and water are mixed to form a gel. The pH is adjusted with the sodium hydroxide solution and Pirlimycin-mannosylated serum albumin is stirred into the gel until uniformly dispersed and the gel is filled into a mastitis syringe.

TABLE 1

2'0 Esters of Pirlimycin

Compound	Time	Concentration*
Bovine mammary gland macrophages		
(a)	15 minutes	8.2
	1 hour	12.0
(b)	15 minutes	4.0
	1 hour	8.5
(c)	15 minutes	15.0
	1 hour	18.0
Rabbit alveolar macrophages		
(b)	15 minutes	6.0
	1 hour	32.0
(c)	15 minutes	30.0
	1 hour	78.0

*expressed as pirlimycin per mg of cell protein

CLAIMS

1. A chemical complex having the formula

LINCOSAMINIDE-RADICAL-CARRIER

wherein LINCOSAMINIDE is an antibiotic lincosaminide; RADICAL is an organic compound attached to a hydroxyl group of the LINCOSAMINIDE by a carboxyl group and attached to the CARRIER by a carboxyl or amino group; and CARRIER is an organic compound capable of transporting or directing said complex to a specific cell type.

2. A chemical complex according to claim 1 wherein said LINCOSAMINIDE is separated from said RADICAL when incubated in aqueous media.

3. A chemical complex according to claim 2 wherein said LINCOSAMINIDE is selected from the group consisting of pirlimycin, clindamycin and lincomycin.

4. A chemical complex according to claim 3 wherein said RADICAL is selected from the group consisting of succinyl, glutaryl, 2,2-dimethylglutaryl, 3,3-dimethylglutaryl, 3-ethyl-3-methylglutaryl, 2-phenylglutaryl, 3,3-tetramethyleneglutaryl, phtalyl, 3-nitrophtalyl, isatoyl, N-methylisatoyl, 1,2-cyclohexanoyl, dimethylmaleyl, maleyl, citraconyl, glycyl, alanyl, leucyl, phenylalanyl, glycylalanyl, undecanoyl, succinylglycyl, and glycylvalylleucylalanylphenylalanyl-glycyl.

5. A chemical complex according to claim 4 wherein said CARRIER is selected from a group consisting of bovine serum albumin, mannosylated bovine serum albumin and rat immunoglobulin G.

6. A chemical complex according to claim 5 wherein said RADICAL is attached to the 2'-hydroxyl group of pirlimycin, clindamycin or lincomycin.

7. A chemical complex according to claim 6 wherein said LINCOSAMINIDE is pirlimycin.

8. A chemical complex according to claim 7 wherein said RADICAL is

a succinyl.

9. Use of a chemical complex having the formula:

LINCOSAMINIDE-RADICAL-CARRIER

wherein LINCOSAMINIDE is an antibiotic lincosaminide; RADICAL is an organic compound attached to a hydroxyl group of the LINCOSAMINIDE by a carboxyl group and attached to the CARRIER by a carboxyl or amino group; and CARRIER is an organic compound capable of transporting or directing said complex to a specific cell type to prepare a medicament for treating bovine mastitis.

10. A use according to claim 9 wherein said LINCOSAMINIDE is separated from said RADICAL when incubated in aqueous media.

11. A use according to claim 10 wherein said LINCOSAMINIDE is selected from the group consisting of pirlimycin, clindamycin and lincomycin.

12. A use according to claim 11 wherein said RADICAL is selected from the group consisting of succinyl, glutaryl, 2,2-dimethylglutaryl, 3,3-dimethylglutaryl, 3-ethyl-3-methylglutaryl, 2-phenylglutaryl, 3,3-tetramethyleneglutaryl, phtalyl, 3-nitrophtalyl, isatoyl, N-methylisatoyl, 1,2-cyclohexanoyl, dimethylmaleyl, maleyl, citraconyl, glycyl, alanyl, leucyl, phenylalanyl, glycylalanyl, undecanoyl, succinylglycyl, and glycylvalylleucylalanylphenylalanylglycyl.

13. A use according to claim 12 wherein said CARRIER is selected from a group consisting of bovine serum albumin, mannosylated bovine serum albumin and rat immunoglobulin G.

14. A use according to claim 10 wherein said RADICAL is attached to the 2'-hydroxyl group of pirlimycin, clindamycin or lincomycin.

15. A use according to claim 14 wherein said LINCOSAMINIDE is pirlimycin.

16. A use according to claim 15 wherein said RADICAL is a succinyl.

17. A use according to claim 8 wherein an effective amount of said chemical complex is from about 0.1 to about 100 mg per kg.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03865

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: A 61 K 47/00, 49/00, 37/00, 31/70

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC4	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category 10	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	EP, A, 0 176 429 (UNIVERSITE PIERRE ET MARIE CURIE) 2 April 1986, see pages 5-6, especially page 5 lines 15-23	1-3
Y	--	4-17
Y	US, A, 4 376 765 (A B L TROUET ET AL.) 15 March 1983, see column 2 - column 3, claims	4,5,12, 13
Y	-- Clinical Chemistry, Vol. 22, No. 6, 1976 T J Gilbertson et al.: "Radioimmunoassay for clindamycin". , see page 828 - page 831 see the whole document	1-8
	--	

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication data of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th February 1989	03. 03. 89

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4 671 958 (J D RODWELL ET AL.) 9 June 1987, see column 8-13, table I-III --	4,12
Y	Chemical Abstracts, vol. 95, 1981, No. 10, 7 September 1981, (Columbus, Ohio, US), Research Products Rehovot Ltd. "Antibiotic formulation containing clindamycin for the treatment of bovine mastitis", see page 342, abstract 86323d, & Israeli 53072, 30 January 1981, Appl. 6 Oct. 1977 --------	9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 88/03865

SA 25495

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/01/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0 176 429	02/04/86	FR-A-B- 2570278	21/03/86
		JP-A- 61143328	01/07/86
		OA-A- 8099	31/03/87
US-A- 4 376 765	15/03/83	BE-A- 882541	16/07/80
US-A- 4 671 958	09/06/87	EP-A- 0088695	14/09/83
		AU-D- 11990/83	15/09/83
		JP-A- 58222035	23/12/83
		CA-A- 1203164	15/04/86
		AU-A- 556446	06/11/86